Calmodulin-dependent multiprotein kinase and protein kinase C phosphorylate the same site on HMG-CoA reductase as the AMP-activated protein kinase

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Calmodulin-dependent multiprotein kinase and protein kinase C phosphorylate and inactivate both intact, microsomal HMG-CoA reductase, and the purified 53 kDa catalytic fragment. Isolation of the single phosphopeptide produced by combined cleavage with cyanogen bromide and Lys-C proteinase reveals that this is due to phosphorylation of a single serine residue near the C-terminus, corresponding to serine-872 in the human enzyme. This is identical with the single serine phosphorylated by the AMP-activated protein kinase. The nature of the protein kinase responsible for phosphorylation of this site in vivo is discussed.

HMG-CoA reductase; Cholesterol synthesis; Protein kinase; Phosphorylation site; Protein phosphorylation

1. INTRODUCTION

The principal regulatory step in the biosynthetic pathway of cholesterol and other isoprenoid compounds, the conversion of HMG-CoA to mevalonate, is catalysed by HMG-CoA reductase [1]. This enzyme is subject to complex multivalent control mechanisms [2], including acute regulation of the activity of the enzyme by reversible phosphorylation [1]. Several protein kinases phosphorylate and inactivate HMG-CoA reductase in vitro, including the AMP-activated protein kinase (formerly termed HMG-CoA reductase kinase), which is itself activated allosterically by 5'-AMP, and by phosphorylation by a distinct kinase [3-5]. The AMP-activated protein kinase is the major Ca^{2+}-independent HMG-CoA reductase kinase in rat liver and may have a general role in the regulation of lipid metabolism [4]. Recently we have demonstrated that the AMP-activated protein kinase phosphorylates a single site on HMG-CoA reductase (corresponding to serine-872 in the human enzyme). This site is highly phosphorylated in intact rat liver under conditions where HMG-CoA reductase is largely in the inactive form [6]. HMG-CoA reductase is also phosphorylated by protein kinase C [7] and two calmodulin-dependent protein kinases from rat brain [8]. One of the latter was apparently identical with calmodulin-dependent multiprotein kinase (calmodulin-dependent protein kinase II) [9], while the other appeared to be a low molecular weight isoform with a different β-subunit [8]. In this study we show that protein kinase C and calmodulin-dependent multiprotein kinase phosphorylate and inactivate HMG-CoA reductase by phosphorylation at the same single site phosphorylated by the AMP-activated protein kinase.

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2. MATERIALS AND METHODS

2.1. *Materials*

Calmodulin-dependent multiprotein kinase was purified from rat brain [10]. Calmodulin purified from sheep brain [11], glycogen synthase purified from rabbit skeletal muscle [12] and the catalytic subunit of protein phosphatase 2A purified from rabbit skeletal muscle [13] were provided by Mr Barry Caudwell Dr Julie Pitcher and Mr Don Schelling respectively in this department. Protein kinase C (mixture of rat brain isozymes) was donated by Dr Peter Parker, Ludwig Institute, London and Dr Alastair Aitken, National Institute for Medical Research, London. The catalytic fragment and intact forms of HMG-CoA reductase were prepared from rats fed diets containing cholestyramine and mevinolin [4,6]. Phosphatidylserine was from Lipid Products, Surrey, UK. Tetradecanoyl phorbol acetate (TPA) and histone H1 (Type III-S) was from Sigma Chemical Co., Poole, UK. Anti-HMG-CoA reductase sera and other materials have been described elsewhere [4,6].

2.2. *Phosphorylation of the catalytic fragment of HMG-CoA reductase*

The catalytic fragment of HMG-CoA reductase (0.1-0.2 mg/ml) was phosphorylated at 30°C in incubations which contained 50 mM Na MOPS, pH 6.5, 4 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (2-5 $\times 10^5$ cpm/nmol by Cerenkov counting) and either calmodulin-dependent multiprotein kinase, 10% (by vol.) glycerol, 0.01 mg/ml calmodulin and 0.5 mM $CaCl_2$, or protein kinase C, 1.25 mg/ml phosphatidyl serine, 1.25 ng/ml TPA, 0.25% (v/v) Triton X-100 and 1 mM $CaCl_2$. Phosphatidylserine, TPA and Triton X-100 were added as mixed micelles [14].

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Measurement of inactivation and phosphorylation of HMG-CoA reductase, and digestion with cyanogen bromide and Lys-C endoproteinase were as described previously [6].

2.3. *Phosphorylation and immunoprecipitation of the native (100 kDa) form of HMG-CoA reductase*

To dephosphorylate HMG-CoA reductase and inactivate endogenous AMP-activated protein kinase, microsomes (20 mg protein/ml) were incubated at 30°C in 20 mM Tris HCl, pH 7.2, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethanesulphonylfluoride (PMSF), 0.1 mM leupeptin, 4 mM dithiothreitol and the catalytic subunit of protein phosphatase 2A. After 15 min, phosphatase activity was blocked with the addition of NaF and Na pyrophosphate to 50 mM and 5 mM respectively, and the microsomes isolated by centrifugation and resuspended in 50 mM MOPS, pH 6.5, 50 mM NaF, 5 mM NaPPi, 4 mM dithiothreitol, 1 mM PMSF, 0.1 mM leupeptin.

Incubation of the microsomes with calmodulin-dependent multiprotein kinase or protein kinase C was then carried out at 20 mg protein/ml in a reaction volume of 250 μl under the same conditions used for phosphorylation of the catalytic fragment, except that 50 mM NaF, 5 mM NaPPi, 1 mM PMSF and 0.1 mM leupeptin were included in the incubation buffers. The reaction was terminated by the addition of EDTA to 17 mM, and intact (100 kDa) HMG-CoA reductase was immunoprecipitated and digested as described in [6].

2.4. *Purification and analysis of phosphopeptides and other analytical procedures*

HPLC purification, sequencing, mass spectrometry and isoelectric focussing of phosphopeptides were as described previously [6]. Protein concentration was measured by the dye-binding method of Bradford [15].

3. RESULTS

3.1. *Phosphorylation and inactivation of HMG-CoA reductase by calmodulin-dependent multiprotein kinase and protein kinase C*

Calmodulin-dependent multiprotein kinase inactivated the intact (97 kDa) form of HMG-CoA reductase, detergent-solubilized from liver microsomes, and this inactivation was totally dependent on the presence of calcium and calmodulin. Purified catalytic fragment of HMG-CoA reductase (53 kDa) was also inactivated with a concomitant incorporation of phosphate (Fig. 1). The initial rate of phosphorylation of the catalytic fragment was about 25% that of glycogen synthase, which is one of the better substrates for the calmodulin-dependent multiprotein kinase [9] (both substrates at 2 μM). As reported for the low molecular weight calmodulin-dependent protein kinase [8], the rate of phosphorylation of the catalytic fragment of HMG-CoA reductase at pH 7.2 was 7-fold lower than at pH 6.5 (not shown).

As previously reported [7], protein kinase C inactivated both the intact microsomal and catalytic fragment forms of HMG-CoA reductase, although the stoichiometry of phosphorylation of the catalytic fragment was low (<0.2 mol/mol) and the initial rate of phosphorylation was only 10% of that of histone H1 (not shown). Cyclic AMP-dependent protein kinase did not phosphorylate HMG-CoA reductase significantly (not shown).

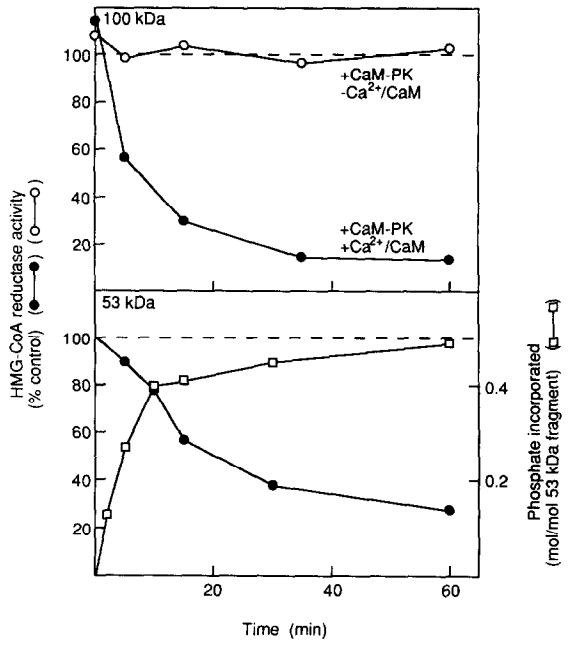

Fig. 1. Phosphorylation and inactivation of HMG-CoA reductase by calmodulin-dependent multiprotein kinase. The top panel shows inactivation of the detergent-solubilized [4] intact enzyme (100 kDa) in the presence (filled circles) and absence (open circles) of Ca^{2+}/calmodulin: results are expressed as a percentage of the activities in a control incubation lacking kinase. The bottom panel shows results with the soluble 53 kDa fragment of HMG-CoA reductase. Activity (filled circles) is expressed as a percentage of the activity of controls lacking Ca^{2+}/calmodulin; open squares show the Ca^{2+}/calmodulin-dependent phosphorylation: a small amount of Ca^{2+}/calmodulin-independent phosphorylation (<0.05 mol/mol) has been subtracted.

3.2. *Analysis of the site phosphorylated on the catalytic fragment of HMG-CoA reductase by calmodulin-dependent multiprotein kinase and protein kinase C*

Five nmol of the catalytic fragment of HMG-CoA reductase was phosphorylated either by calmodulin-dependent multiprotein kinase to a stoichiometry of 0.5 mol phosphate/mol fragment (producing an inactivation of 63% compared to controls lacking kinase), or by protein kinase C to a stoichiometry of 0.2 mol phosphate/mol fragment (23% inactivation). Digestion with cyanogen bromide and Lys-C endoproteinase and analysis by reversed phase HPLC demonstrated that, following phosphorylation by either kinase, a single phosphopeptide was produced which eluted at ~23 min, on the acetonitrile gradient used, identical to that produced after phosphorylation by the AMP-activated protein kinase [6] (Fig. 2). The phosphopeptides were pure after this single run, and had the amino acid sequence $V_{113}H_{17}N_{57}R_{21}S_7K_{33}$ (calmodulin-dependent protein kinase) and $V_{66}H_{12}N_{29}R_9S_{24}K_{15}$ (protein kinase C) [subscripts donate the pmol of phenylthiohydantoin derivative detected at each cycle]. In the case of the calmodulin-dependent protein kinase, fast atom bom-

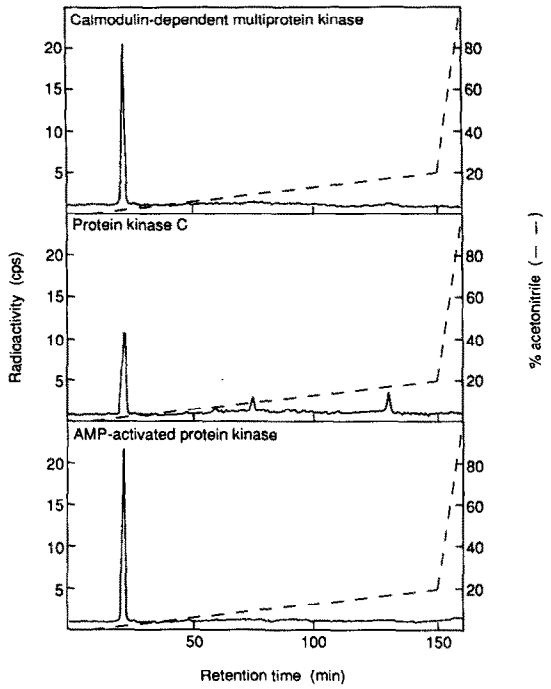

Fig. 2. Reversed-phase HPLC analysis of cyanogen bromide/Lys-C proteinase peptides derived from the 53 kDa fragment of HMG-CoA reductase, after phosphorylation using [γ-^{32}P]ATP and three different protein kinases. The continuous line shows radioactivity detected using an on-line monitor.

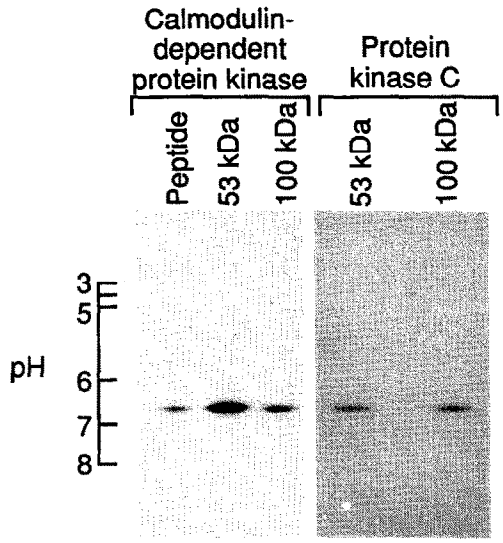

Fig. 3. Isoelectric focussing of cyanogen bromide/Lys-C proteinase peptides derived from HMG-CoA reductase which had been phosphorylated using [γ-^{32}P]ATP and calmodulin-dependent multiprotein kinase or protein kinase C. The photograph shows an autoradiogram of the dried gel, with the pH scale being estimated using coloured marker proteins (BDH). HMG-CoA reductase was either phosphorylated in microsomes and isolated using immunoprecipitation (100 kDa), or the catalytic fragment was purified to homogeneity and then phosphorylated (53 kDa). 'Peptide' represents the VHNRSK peptide purified as for the upper panel of Fig. 2.

bardment-mass spectrometry of the peptide demonstrated that the MH^+ ion had the expected mass of 820. After ethanethiol derivatization of the peptide, which converts phosphoserine to the novel, stable amino acid, *S*-ethylcysteine, the phenylthiohydantoin derivative of *S*-ethylcysteine was detected at cycle 5 during sequencing, conclusively demonstrating that this serine was the phosphorylated residue.

3.3. *Analysis of the site phosphorylated on native microsomal HMG-CoA reductase*

Incubation of crude microsomes (pre-treated with protein phosphatase 2A) with unlabelled ATP and either calmodulin-dependent protein kinase or protein kinase C produced an inactivation of HMG-CoA reductase of 55% and 30% respectively compared to controls incubated without kinase (not shown). HMG-CoA reductase was phosphorylated using [γ-^{32}P]ATP under the same conditions, and then immunoprecipitated. Polyacrylamide gel electrophoresis of the immunoprecipitates in sodium dodecyl sulphate showed that intact (97 kDa) HMG-CoA reductase was the only ^{32}P-labelled polypeptide in the precipitate following phosphorylation by either kinase. Digestion with cyanogen bromide and Lys-C endoproteinase produced a single phosphopeptide which comigrated on isoelectric focussing with the VHNRSK peptide produced following phosphorylation of the catalytic fragment (Fig. 3), confirming that this is the only site on intact HMG-CoA reductase phosphorylated by calmodulin-dependent multiprotein kinase or protein kinase C.

4. DISCUSSION

In this study we have established that calmodulin-dependent multiprotein kinase and protein kinase C inactivate HMG-CoA reductase via phosphorylation of the same single site that is phosphorylated by the AMP-activated protein kinase. This regulatory site, which corresponds to serine-872 in the human sequence [6], is located close to the C-terminus of HMG-CoA reductase, and the primary sequence of this region in the rat enzyme is shown in Fig. 4. We have suggested that recognition by the AMP-activated protein kinase may require hydrophobic residues on either side of the phosphorylated serine residue, which may be located on a β-turn [6]. Sites phosphorylated by calmodulin-dependent multiprotein kinase normally have an arginine on the N-terminal side (at -3) of the phosphorylated residue [9, Fig. 4]. In the case of HMG-CoA reductase the residue at -3 is a histidine, although there is an arginine residue at -1. It is possible that histidine can replace arginine in the recognition site as long as it carries a positive charge. This might explain the higher rate of phosphorylation of HMG-CoA reductase at pH 6.5 compared with pH 7.2. Protein

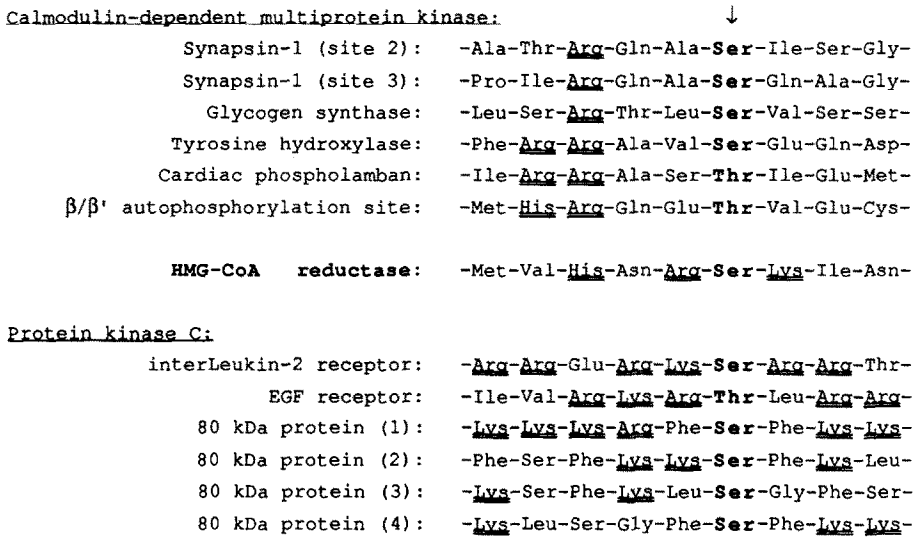

Fig. 4. Comparison of the sequence around the site phosphorylated on HMG-CoA reductase with those on other substrates for calmodulin-dependent multiprotein kinase (top) and protein kinase C (bottom). Basic residues, including histidine, are underlined; phosphorylated serine/threonine residues are marked with an arrow and bold type.

kinase C prefers sites that have one or more basic residues (arginine or lysine) on the N- and C-terminal side of the phosphorylated residue [16, Fig. 4], and in HMG-CoA reductase this is satisfied by an arginine at -1 and lysine at $+1$. Cyclic AMP-dependent protein kinase, which does not phosphorylate HMG-CoA reductase significantly, has a minimum requirement for an arginine at $-2/-3$ [17], not found at this site.

We have demonstrated recently that HMG-CoA reductase is almost completely inactivated and highly phosphorylated at the C-terminal site (corresponding to serine-872 in the human enzyme) when rats are killed and the livers removed by dissection. Under these conditions, it is likely that the AMP-activated protein kinase is responsible for phosphorylation of HMG-CoA reductase [6]. However, phosphorylation and inactivation by three protein kinases in vitro means that HMG-CoA reductase, and hence cholesterol and isoprenoid biosynthesis, can potentially be regulated by diverse signals in vivo. Since the three protein kinases phosphorylate the same site, we cannot establish by site analysis which is responsible for phosphorylation in vivo. One approach to examine whether calmodulin-dependent multiprotein kinase and/or protein kinase C might be physiological regulators is to stimulate these kinases pharmacologically in isolated cells and examine the effect on phosphorylation of HMG-CoA reductase. In isolated hepatocytes, phorbol esters or synthetic diacylglycerols which activate protein kinase C do not cause inactivation of HMG-CoA reductase (V.A. Zammit and A. Caldwell, personal communication). Although hormones or ionophores which elevate intracellular calcium do cause inactivation [18], this is not blocked by calmodulin antagonists (V.A.Z. and A.C., personal communication). These results would argue against a role for protein kinase C or calmodulin-dependent protein kinases in the regulation of HMG-CoA reductase in these cells. However, now that the location of the site of phosphorylation has been identified, it will be possible to examine the effects of these agents on phosphorylation of HMG-CoA reductase more directly.

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